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Transmission of HIV-1 infection between trophoblast placental cells and T-cells take place via an LFA-1-mediated cell to cell contact

Rebeca Alonso Arias, Laura Díaz Muñoz, and M[a] Angeles Muñoz-Fernández*

Department of Immunology, Hospital General Universitario Gregorio Marañón, Madrid, Spain

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Abstract

HIV-1 vertical transmission is thought to mainly take place by virus crossing the placental barrier. However, the mechanism by which HIV-1-infects placental cells remains to be elucidated. We have found that purified cytotrophoblasts as well as trophoblastic cell lines are susceptible to infection by different HIV-1 isolates as detected by DNA-PCR and release of infectious virus, although with very low efficiency. Purified trophoblast or trophoblastic cell lines express low levels of chemokine receptors CCR-5 and CXCR-4 but not CD4 on the cell surface. To test if those molecules were used as receptors for HIV-1 infection, placental cells were pretreated with antibodies to CD4, CC-chemokines, C-X-C chemokines. None of those treatments inhibited HIV-1 infection. In contrast, we have found that HIV-1 infection of placental cells was increased in cocultures of infected T-cell blasts and placental cells. More interestingly, antibodies to β_2 integrins and to LFA-1 were able to significantly block infection of placental cells. Cell surface expression of ICAM-1, an adhesion molecule involved in attachment of leukocytes to placenta, was upregulated in HIV-1-infected placental cells. Placental cells were able to transfer HIV-1 infection to T-cell blasts. This transmission required cell to cell contact and was also inhibited by anti-LFA-1 antibodies. In summary our results suggest that placental trophoblast could be infected by HIV-1 by a mechanism involving T cell to placental contact. Moreover, placental infection enhanced ICAM-1 expression and leukocyte adherence, an event which was required to transfer HIV-1 infection to T cells. This provides an explanation of the virus passing through the placental barrier during in utero HIV-1 vertical transmission.

Keywords: HIV; Children; Vertical transmission; Adhesion molecules

Introduction

Vertical transmission of HIV-1 is mainly produced at three different periods: antenatally, peripartum or postnatally (Pizzo and Butler, 1991). Most efforts have been conducted to prevent the antenatally virus transmission. However, it has been estimated that around 30% of the vertically HIV-1 infected children acquire the infection in early pregnancy, in utero (Alonso et al., 1998). Clinically, children infected in utero show a distinctive pattern of AIDS progression and/or opportunistic infections characteristic of AIDS (Mofenson and Wolinsky, 1993). In this case, the virus could pass through placental barrier from maternal to fetal blood. In fact, the presence of virus in tissues obtained from fetus in different gestational ages and in different placental tissues has been demonstrated (Mattern et al., 1992; Papiernik et al., 1992; Soeiro et al., 1992). Thus, the infection of placental cells could be directly involved in the entry of the virus to the fetal blood.

On the other hand, placental inflamation, as chorioamnionitis, has been found more often in HIV-1 infected pregnant women than in healthy women. This inflamation could produce placental damage, which in turn may favor the virus entering placental into tissues (Schwartz and Nahmias, 1991; Wabwire-Mangen et al., 1999). In addition, inflammatory cytokines are known to increase HIV-1 replication (Alonso et al., 2000; Munoz-Fernandez et al., 1997; Poli et al., 1994). Inflammatory cytokines may also lead to adverse effects on the placental barrier by damaging the placental

* Corresponding author. Department of Inmunología, Hospital General Universitario Gregorio Marañón, c./Doctor Esquerdo 46, 28007-Madrid, Spain. Fax: +91-586-8018.

E-mail address: Mmunoz@cbm.uam.es (M.A. Muñoz-Fernández).

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trophoblast (Raghupathy, 1997). In all these instances, the exposure of susceptible placental cells to the virus and subsequent infection could be increased. On the other hand, there is little direct evidence about placental cell infection. Viral antigens and nucleic acids have been identified in placental tissue from HIV-1 infected women by immunocytochemistry and in situ hybridization (Chandwani et al., 1991; Lewis et al., 1990; Mattern et al., 1992; Maury et al., 1989; Moussa et al., 1999; Sheikh et al., 2000). However, the type and distribution of the infected placental cells is not clear. The trophoblast cellular layer is in direct contact with maternal blood and it would be a good candidate to become infected by HIV-1 and to transmit the infection to the fetus. This hypothesis has caused some controversy and contradictory results have been reported about both susceptibility to placental cells HIV-1 infection (David et al., 1992; Douglas et al., 1991; Mano and Chermann, 1991; Zachar et al., 1991a) as well as their ability to express the viral receptor CD4 in their cell surface (Lairmore et al., 1993; Maury et al., 1989; Zachar et al., 1991b).

Here, we have studied the susceptibility of trophoblast cells to in vitro HIV-1 infection and the viral ability of HIV-1 to replicate in this cellular type. Furthermore, we have studied the mechanism of viral infection including expression of viral receptors and coreceptors of trophoblast cells.

Our results indicate that placental trophoblast cells derived from normal placental tissue or transformed trophoblastic cell lines could be infected by several different HIV-1 isolates although virus did not seem to use the CD4 receptor molecule or chemokine receptors for entry. More interestingly, HIV-1 infection was greatly enhanced by coculturing placental cells with HIV-1 infected PBMC in a LFA-1 (CD11a) dependent manner. Besides, HIV-1 infection induced an increase in ICAM-1 (CD54) expression in placental cells and adhesion of PBMC to the infected cells. This adhesion to transfer much more efficiences the virus to T cells. Moreover, antibodies against LFA-1 or β2-integrins significantly inhibited both T-cell adhesion to infected placental cells and HIV-1 infection. Taken together, our results may provide an explanation to the mechanism by which HIV can cross the placental barrier and infect the fetus in utero.

Results

Susceptibility of placental cells to HIV-1 infection

In order to determine the ability of HIV-1 to infect placental tissue, we selected four HIV-1 strains with different cellular tropism. Thus, laboratory isolates HIV-$1_{NL4.3}$ (lymphotropic X4), HIV-1_{BaL} (macrophage-tropic R5), as well as primary isolates HIV-1_{1498I} (SI and lymphotropic X4), and HIV-1_{2069I} (macrophage-tropic R5) obtained from infected pregnant women in our laboratory were employed. Purified cytotrophoblast as well as placental cell lines JAR and JEG-3 were used, since those two cell lines show many characteristics of placental trophoblast (Kohler and Bridson, 1971; Pattillo and Gey, 1968). Cells were cultured with these different viral strains for 4 h and then intensely washed to remove non-internalized virus. In those conditions proviral DNA could be detected up to 6 days after infection in any of the cellular types infected with any of the four viral strains (Fig. 1A), indicating HIV-1 entry and integration. To discriminate between latent infection and low viral replication, we quantified viral load in the culture supernatant, which is a more sensitive technique than p24 antigen. Viral load could be detected in the supernatant of infection JAR cell line, 4 days after infection with any of the four viral isolates. A representative experiment is shown in Fig. 1B. Similar results were obtained with purified cytotrophoblast and the JEG-3 cell line (not shown). Those results indicate that all strains, regardless of their tropism and phenotype, replicated at detectable although variable levels in placental cells. To confirm active replication we examined the kinetics of infection in the JAR cell line, quantifying the viral load levels in the culture supernatant at different time-points after infection with the primary isolates HIV-1_{1498I} (X4). The results showed a significant increasing of viral load from 0 to 12 h peaking at 24 h after the infection. This was followed by a decrease, suggestive of a persistent infection, with low levels of viral replication (Fig. 1C). This was also suggested by the fact that no cytopatic effect was observed, as the viability of HIV-1 infected placental cells was always greater than 95% over the entire 6 day culture period. Moreover, PCR with primers of human Alu and HIV-1 sequences gave positive results (data not shown).

Mechanism of entry of HIV-1 into placental cells

CD4 is the main HIV-1 receptor in lymphocytes, although the chemokine receptors CXCR4, CCR5, and CCR3, among others, have been found to act as HIV-1 coreceptors (Chandwani et al., 1991; Hesselgesser et al., 1997). Since placental cells could be infected by HIV-1, we investigated whether the virus could use these receptors and/or coreceptors to enter the cells. We could not detect any CD4 positive cells by flow cytometry in purified trophoblasts and the JAR cell line (not shown). To confirm this we performed RT-PCR for CD4 mRNA, which is a more sensitive technique, in JAR cell line and in primary trophoblast cultures, before or after HLA negative selection. CD4 mRNA could be amplified in placental cells before purification by negative selection but not in purified trophoblasts (Fig. 2A). To further discard the implication of CD4 in HIV-1 entry in the trophoblastic cells, neutralization experiments with anti-CD4 antibodies on HIV-1 infection were carried out. The cell line JAR was incubated with two anti-CD4 (OKT4A and Leu3a) monoclonal antibodies and after washing the cells HIV-$1_{NL4.3}$ (X4) and HIV-1_{BaL} (R5)

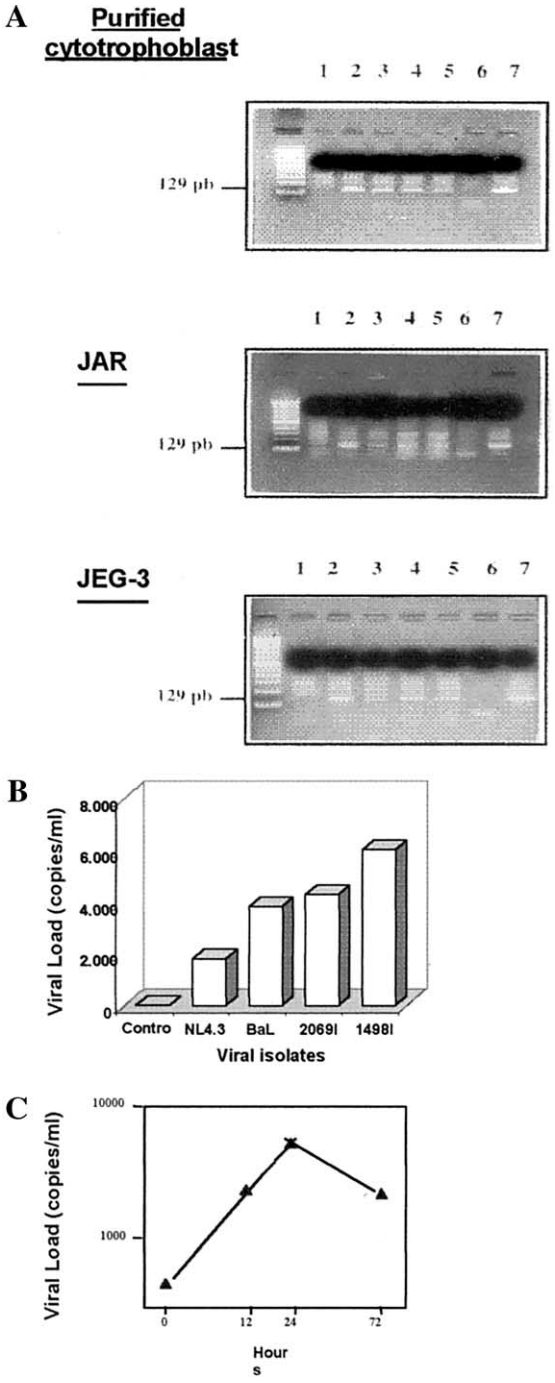

Fig. 1. Infection of placental cells by different HIV-1 isolates. Purified cytotrophoblast cells and JAR and JEG-3 cell lines were infected with 0.1 m.o.i. of the primary isolates HIV-1_{2069I} (R5) and HIV-1_{14981} (X4), as well as with the established strains HIV-$1_{NL4.3}$ (X4) and HIV-1_{BaL} (R5) or mock infected with heat inactivated HIV-$1_{NL4.3}$ (10 m.o.i.). (A) HIV-1 viral DNA was detected in the cell cultures by nested PCR of the *pol* region of HIV-1 6 days after infection. Lane 1, mock-infected cells; lanes 2–5, placental cells infected with HIV-$1_{NL4.3}$, HIV-1_{BaL}, HIV-1_{2069I} and HIV-1_{1498I}, respectively; lane 6, negative control; and lane 7, positive control of an infected T-cell line. (B) Viral load was quantified in the supernatants of cultures of JAR cells by RT-PCR 4 days after infection, with the four isolate. (C) Kinetics of virus release to supernatants. Viral load was quantified by RT-PCR in the supernatant of JAR cell lines at the indicated times hours after infection with HIV-1_{1498I}.

virus were added. PBMC were used as a positive control. No blocking effect of anti-CD4 monoclonal antibodies could be detected in the JEG-3 cell cultures, while the inhibition reached 99% in PBMC (Fig. 2B). Similar results were obtained with purified cytotrophoblast and the JEG-3 cell line (data not shown).

On the other hand, low levels of CXCR4 expression was detected by flow cytometry of JAR cells and JEG-3 cells (Fig. 3A). No CCR5 was detected by flow cytometry in those cell lines (data not shown). CXCR4, CCR3 and CCR5 mRNA expression was also analysed by RT-PCR. Primary purified trophoblast cells expressed CCR5 and CXCR4 mRNA whereas JAR and JEG-3 cell lines expressed CXCR4, CCR5, and CCR3 mRNA (Fig. 3B). Since mRNA

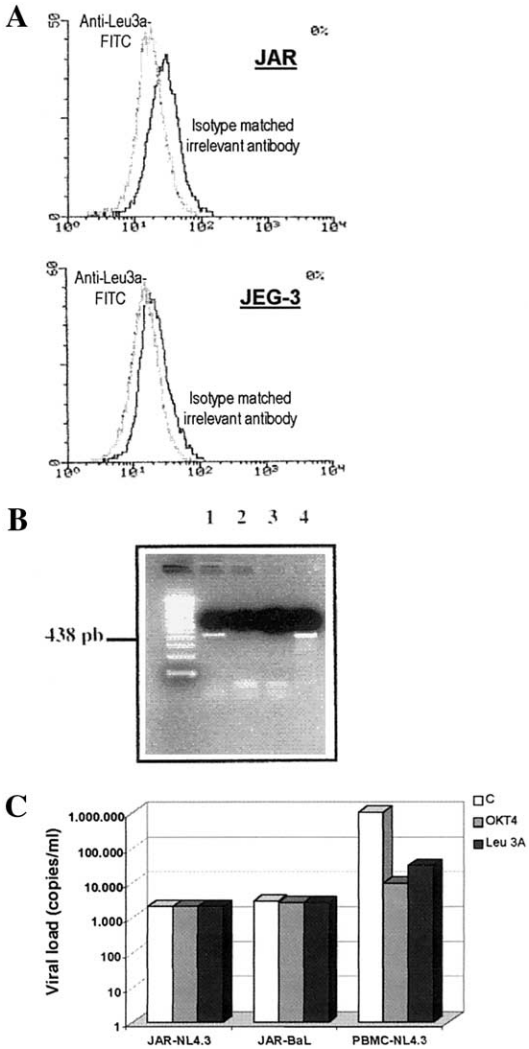

Fig. 2. Role of CD4 in HIV-1 infection of placental cells. (A) CD4 mRNA expression, CD4 mRNA was extracted from placental cells and detected by RT-PCR. Lane 1, primary trophoblasts before HLA negative selection; lane 2, primary purified trophoblasts after HLA negative selection; lane 3, JAR cell line; and lane 4, positive control. (B) Effect of anti-CD4 antibodies. JAR cell line or PHA activated PBMC were incubated with anti-CD4, OKT4A and Leu3a mAb before infection with HIV-$1_{NL4.3}$ or HIV-1_{BaL}. Viral load was determined in the supernatant 4 days later.

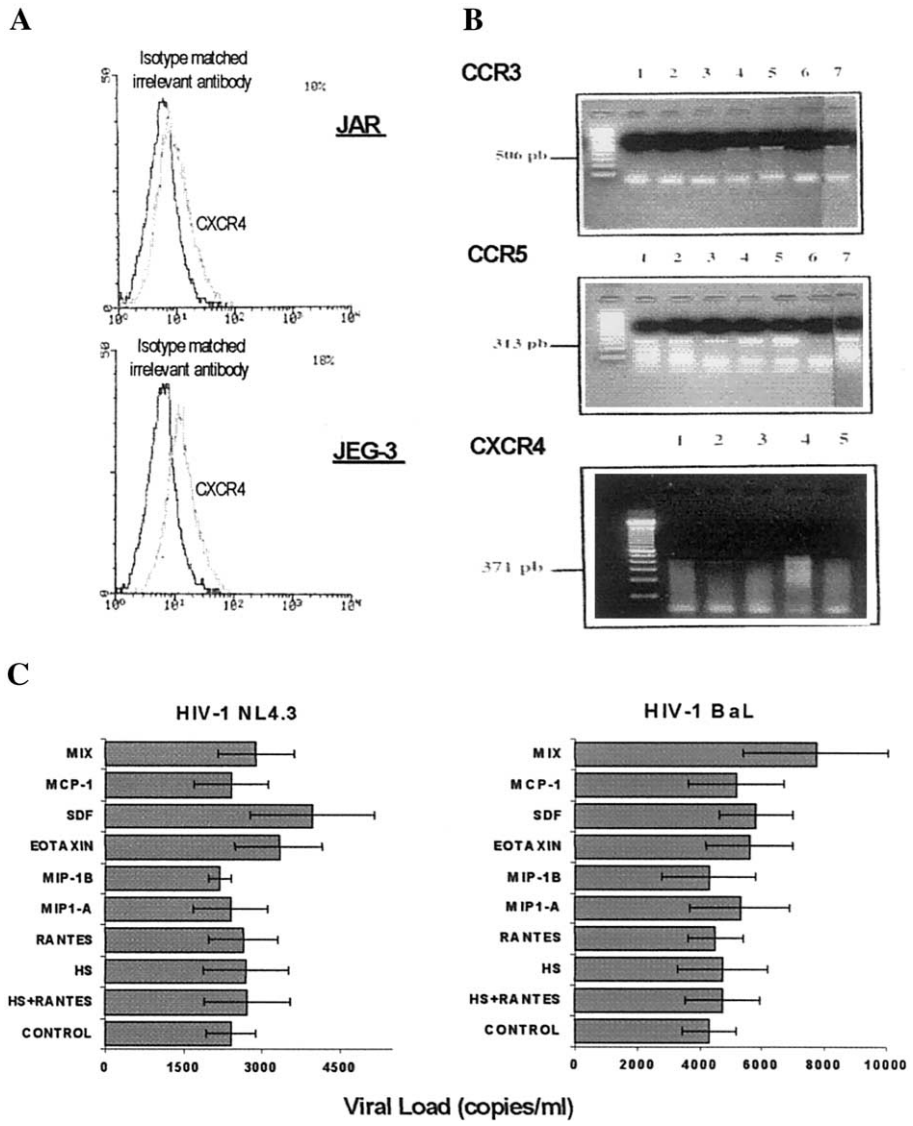

Fig. 3. Chemokine receptor expression in placental cells. (A) CXCR4 expression detected by flow cytometry. Staining with CXCR4 or an isotype matched irrelevant antibody in JAR and JEG-3 cell lines. (B) Expression of CCR3 and CCR5 mRNA. mRNA was extracted from placental cells and detected by RT-PCR. Lanes 1 and 2, purified cytotrophoblast; lane 3, non-purified syncitiotrophoblasts; lane 4, JAR cell line; lane 5, JEG-3 cell line; lane 6, negative control; lane 7, positive control (T cells for CXCR4 and monocyte/macrophagocytes for CCR3 and CCR5). Expression of CXCR-4 mRNA. Lanes 1 and 2, purified cytotrophoblast; lane 3, JAR cell line; lane 4, JEG-3; and lane 5, negative control. (C) Effect of neutralizing chemokine receptors on HIV-1 infection of placental cells. JEG-3 cell line was incubated with the chemokines RANTES, MIP-1α, MIP-1β, Eotaxin, SDF-1 or a mix of all of them 2 h at 37°C. JEG-3 cell line was also incubated with HS or HS + RANTES. Then, they were infected with HIV-$1_{NL4.3}$ or HIV-1_{BaL}, and viral load was quantified in the supernatant 3 days later by RT-PCR. MCP-1 was added as a negative control. The results are the mean of three different experiments.

for all three chemokine receptors was detected in the JEG-3 cell line, we performed neutralisation experiments with different chemokines using $HIV_{NL-4.3}$ or HIV-1_{BaL} isolates for infection in placental cells using this cell line. Neither the infection with HIV-1_{BaL} (R5) nor with HIV-$1_{NL-4.3}$ (X4) was significantly inhibited by any chemokine alone (MIP-1α, MIP-1β, RANTES, Eotaxin, SDF-1α) or by a combination of all of them (Fig. 3C). The small effect observed with MIP-1β shown in Fig. 3 was not reproducible and not statistically significant. In parallel control cultures, SDF-1 inhibited the entry of X4 virus in T cells and MIP-1α, MIP-1β and RANTES inhibited the entry of R5 virus in macrophages, as expected (data not shown). Moreover, the addition of heparin sulfate (HS) known to increase RANTES inhibition did not alter HIV-1 infection of JAR cells by itself or potent RANTES effect.

Adherence of HIV-1 infected T cell blast enhances infection of placental cells

The above results indicate that placental cells could be infected by HIV-1 albeit the extent of infection was rather low. Thus, we tested if infected T-cell blasts, which are in close contact to the trophoblast in the infected pregnant

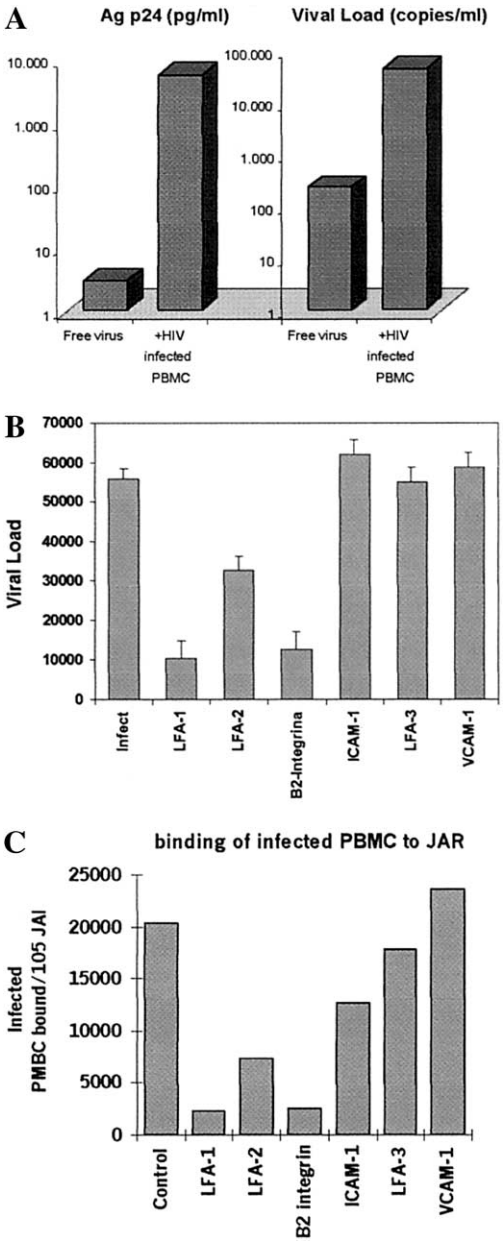

Fig. 4. Transfer of HIV-1 infection from T cell infected blasts to placental cells. (A) Infected PHA activated T-cell blasts can transfer HIV-1 infection to placental cells. JAR monolayers were cultured for 6 h with HIV-1 infected PHA activated PBMC or with free HIV-$1_{NL4.3}$ virus for 6 h. Then the monolayers were extensively washed (10–20 times until no lymphocytes or other cells could be detected in the cultures). Three days later viral load was evaluated in the supernatant. (B) Effect of antibodies to integrins in HIV-1 infection or placental cells by infected PBMC. JAR cells were treated or not (control) with the indicated antibodies (50μg/ml) for 1 h prior adding HIV-1 infected T-cell blasts. Six h later cultures were washed and infection evaluated as above. (C) Effect of antibodies to integrins in the adhesion of infected T-cell blasts to JAR monolayers. Cells were incubated as above and the number of cell bound to JAR monolayer after 6 washes was evaluated as described in materials and methods.

women, can transfer more efficiently HIV-1 infection to placental cells. For this, infected T-cell blasts were cultured for 6 h with JAR monolayer cells. Then, monolayers were extensively washed and viral load determined 3 days later. As shown in Fig. 4A, the infection of placental cells by infected T-cell blasts was more than 2 logs higher than when free virus was used. Moreover, we could detect p24 antigen in the supernatant of JAR cells cocultured with infected PBMC in contrast to cells infected with free virus (not shown). To confirm that the virus was produced by JAR cells and not by contaminating residual cells, we evaluated the number of remaining attached PBMC to JAR monolayer by counting radioactivity of T-cell blasts labelled with [^{3}H]thymidine prior infection. We could not detect any T cells that remained bound. This strongly suggests that the virus production observed came from infected JAR. The above results suggested that infected T-cell blasts adhere to placental cells and transfer the HIV-1 directly to them. To further confirm the role of T cell adhesion in transmitting the infection, JAR monolayer cells were incubated with monoclonal antibodies against cell surface molecule known to be involved in cell to cell contact. As shown in Fig. 4B, the addition of anti-LFA-1 or the β2 integrins monoclonal antibodies prior to and during the coculture period greatly decreased HIV-1 infection of placental cells. Antibodies to LFA-2 had same effect whereas anti-LFA-3 was basically ineffective. Interestingly, the amount of inhibition of viral infection measured in the same cultures after extensive washing to remove all bound lymphocytes correlated with the ability of those monoclonal antibodies to prevent firm adhesion of infected T-cell blasts to JAR monolayers (Fig. 4C).

HIV-1 infection induces ICAM-1 in placental cells

Due the role of LFA-1 in T-cell blast binding to JAR cells, we analysed the expression of LFA-1 counterreceptor (ICAM-1) on JAR cells before or after HIV-1 infection. Uninfected JAR cells expressed ICAM-1 but HIV-1 infection resulted in an enhanced expression of ICAM-1 both in percentage of positive cells and fluorescence intensity (Fig. 5). Interestingly, this effect seems to require viral replication, since treatment of JAR cells with 100-fold more heat inactivated virus had no effect on ICAM-1 expression.

Infected placental cells transfer HIV-1 to T-cells through cell to cell contact

To address if infected placental cells can also transfer the infection to PBMC, JAR cells previously infected with free different viral isolates were cocultured with activated PHA blasts and infection was quantified in the T cells by measurement of p24 antigen. Detectable levels of p24 antigen that increased over time were found in T-cell cultures (Fig. 6A). Since infected JAR cells do not release enough virus to be detected by measuring p24 antigen levels, it is likely that the virus detected came from infected PHA blasts. Interestingly, when we repeated the same experiment using transwells to separate the JAR cell HIV-1-infected line from the PBMC, a decrease in more of 4 $\log_{10}$ in the virus titers by

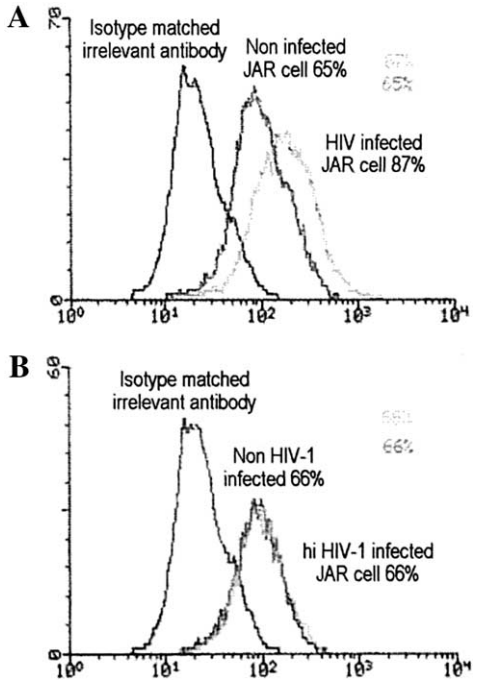

Fig. 5. Induction of ICAM-1 adhesion molecule by HIV-1 infection in placental cells. JAR cell line was infected with (0.1 m.o.i.) of the primary HIV-1_{1498} isolate, or mock infected with heat inactivated (hi) HIV-1_{1498I} (10 m.o.i.). Three days after infection, cells were collected and stained with ICAM-1 specific monoclonal antibody or with an isotype matched irrelevant antibody. Cell surface expression was detected by flow cytometry. Shown inside the panels are the percentages of positive cells with respect to the irrelevant matched monoclonal antibody.

either p24 (Fig. 6B) or in viral load (not shown) was observed. This indicates that cell contact was required to transfer the infectious virus to PBMC.

Next we tested if T-cell blasts can bind to the placental cells. For this 2×10^6 T-cell blasts activated PBMC cells were added to 10^6 JAR cells cultured for 6 h and then washed 6 times. A substantial number of lymphocytes can be observed bound HIV-1-infected JAR monolayerss either by Normaski microscopy (Fig. 7A) or by staining with anti-CD45 (Fig. 7B). Since anti-CD45 is not expressed on JAR cells, the later method only detected bound PBMC cells.

The results indicate so far the transfer of infection virus to placental cells to PBMC was dependent on cell contact and that ICAM-1 was increased in HIV-1 infected placental cells. In order to study the influence of adhesion molecules on the process, we tested the effect of several monoclonal antibodies to cell adhesion molecules on the transfer of infection to PBMC. The addition of human monoclonal antibodies to LFA-1, the ligand of ICAM-1, significantly prevented both adhesion (Fig. 7C) and the transfer of HIV-1 infection to PBMC (Fig. 7D). Moreover, a monoclonal antibody against the common subunit of the β_2 integrins also inhibited viral infection and cell adhesion. In contrast, monoclonal antibodies to other cell surface molecules affected neither infection nor adhesion (Figs. 7C, D).

Discussion

HIV-1 vertical transmission has been related with immunological and clinical characteristics of the carrier mothers as well as with the virological characteristics of the transmitting virus (Resino et al., 2000). Moreover, HIV-1 fetal infection in utero could involve passing of virus or virus infected cells through the placental barrier to the fetal compartment (Aplin, 1991). In this regard, viral gene expression in chorionic membranes of HIV-1 infected pregnant women has been reported (Chandwani et al., 1991; Maury et al., 1989; Sheikh et al., 2000; Wabwire-Mangen et al., 1999). Nonetheless, whether placental tissue is actually infected and the origin and location of infected placental cells remained unclear. The placental barrier is mainly composed of syncytiotrophoblasts, acting as a physical barrier between maternal and fetal blood and providing an immunological protection against transfer of microbial pathogens and/or maternal leukocytes from the mother to the fetal tissues. Since fetal syncytiotrophoblasts are in direct contact with maternal blood, maternal HIV-1 could vertically infect the

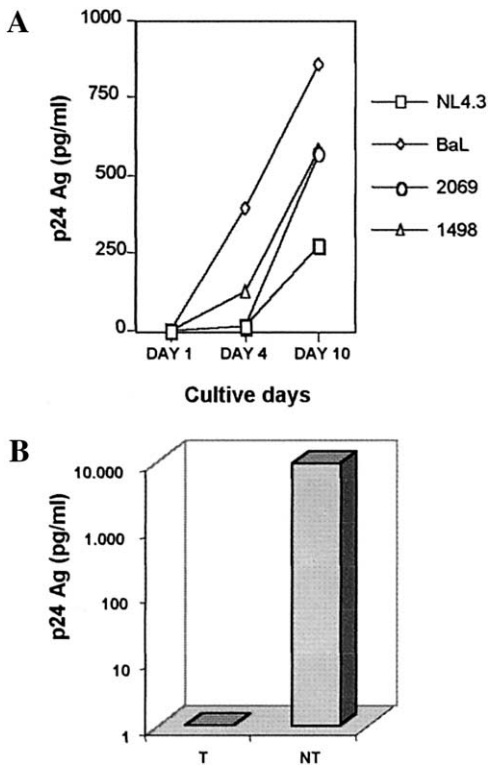

Fig. 6. Infection of T cell blasts by HIV-1-infected placental cells. A) Kinetics of HIV-1 replication in infected placental cells-PBMC coculture. PHA activated PBMC were cultured for 6 h with JAR cell line cultures infected with any of the four viral isolates (HIV-$1_{pNL4.3}$, HIV-1_{BaL}, HIV-1_{2069I}, HIV-1_{1498I}) and then extensively washed away and PBMC collected. After 1, 4, and 10 days virus production was quantified by measuring Agp24 in the supernatant of those T-cell cultures. B) Requirement of physical contact between HIV-1_{1498}-infected placental cells and PBMC to transfer the infection. Transwells were used (T) or not (NT) to separate the JAR cell HIV-1 infected cells from PBMC during the 6 h coculture. Four days later virus production was quantified.

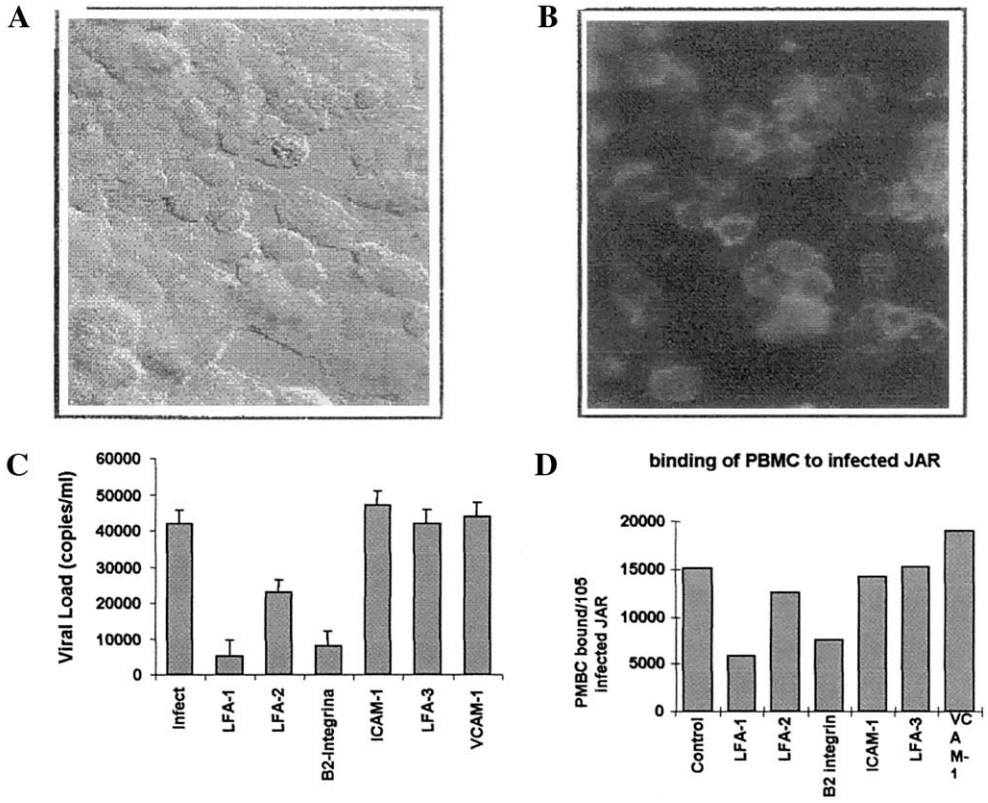

Fig. 7. Binding of T-cell blast to HIV-1 infected placental cells and transmission of viral infection. (A & B) Adherence of PBMC to infected placental cells. JAR cells (10^5 cell/well) were cocultured with PHA-stimulated T-cell blast (2×106/well) during 4 h. Then, the cells were washed 3 times, fixed, and stained with a monoclonal antibody anti-CD45-FITC. (A) A representative optical field by Normarski microscopy. (B) A representative field under fluorescence microscope. (C & D). Effect of antibodies to integrins in the binding of T-cell blasts to infected JAR cells and transmission of HIV-1 infection. PHA blasts were added to infected JAR cultures in absence (control) or in presence of the indicated monoclonal antibodies for 6 h. (C) PBMC were washed away completely from the plate after the coculture period, plated at 10^6/ml and viral load measured 3 days later. (D) Finally, bound cells were evaluated after 6 washes as described in Materials and Methods.

fetus using these cells as a carrier. However, the susceptibility of the syncytiotrophoblast to HIV-1 infection is controversial (David et al., 1992; Douglas et al., 1991; Douglas and King, 1992).

Here, we have studied the susceptibility to HIV-1 infection of primary highly purified trophoblast cultures, and of the human choriocarcinoma cell lines JAR and JEG-3. These cell lines express many characteristics of placental trophoblast (Kohler and Bridson, 1971; Pattillo and Gey, 1968). Our results indicate that both the purified trophoblast and JAR and JEG-3 cell lines could be infected by HIV-1. Moreover, the HIV-1 infection of placental cells was not limited to just one particular viral strain. Interestingly, virus isolated from HIV-1 infected women at delivery, HIV-1_{2069I} R5 and HIV-1_{1498I} X4, had higher levels of replication in placental cells than laboratory adapted viral strains. None of the viral strains induced cytopathic effect in the placental cells or cell lines after 6 days of infection (data not shown), as it has already been reported by other investigators (Fazely et al., 1995). After an initial increase in viral load the infected cells continuously released low levels of virus that remained constant through the cultures suggestive of persistent infection.

Previous studies on HIV-1 infection of trophoblast with free virus have shown low levels of infection (David et al., 1992; Mano and Chermann, 1991; Zachar et al., 1991a), while other authors have reported no infection at all (Douglas et al., 1991). These differences could be attributed to the method of detection. By electron microscopy, the most sensitive method to detect virions, HIV-1 infection and rapid replication have been observed (Grewe et al., 1990; Pauza et al., 1988; Stein et al., 1987). We used a very sensitive method to detect virus, viral load quantification by ultrasensible RT-PCR, that substantially increases during the first 24 hours. We also detected proviral DNA by PCR with specific primers. Both are clearly indicative of viral infection of placental cells.

To investigate the putative mechanism used by the cell-free virus to enter the placental cells we analysed CD4 receptor and CCR coreceptor expression in the trophoblast cells, and in JAR and JEG-3 cell lines by flow cytometry and/or RT-PCR. None of the cells express CD4 by flow cytometry, whereas CD4 mRNA was only found in JEG-3 cell line and in poorly purified but not in highly purified trophoblasts. CD4-mRNA expression in partially purified trophoblast most likely derives from contaminating of Hof-

bauer cells (Maury et al., 1989; Sheikh et al., 2000). In agreement with our results, lack of CD4 mRNA detected by northern blot in the purified trophoblasts has been reported in other works (Lairmore et al., 1993; Zachar et al., 1991a). Furthermore, virus entry through CD4 is highly unlikely since anti-CD4 antibodies did not block HIV-1 infection in contrast to T cells.

The trophoblast cells as well as the JAR and JEG-3 cell lines express CXCR4 and CCR5 mRNA whereas CCR3 was only detected in both cell lines but not in the purified trophoblast cells. Furthermore, low CXCR4 expression by flow cytometry was observed in a small percentage of JAR and JEG-3 cell lines. However, our results clearly demonstrated that viral infection of those placental cell line could not be prevented by blocking the above mentioned HIV-1 coreceptors with their corresponding chemokines. Although it is well known that HIV-1 infects T helper lymphocytes and monocytes/macrophages via CD4 receptor, HIV-1 tropism could be wide. Thus, HIV could infect different tissues in vivo (colon, retina and cervix) as well as CD4 negative cells in vitro (glioma, neuroblastoma, rabdomiosarcoma, hepatocellular carcinoma, fibroblasts, and CD34+ bone marrow precursors) (Li et al., 1990; Obregón et al., 1999). These results are consistent with different infection mechanisms, which could imply either a surface protein different from CD4 or a protein-independent endocytosis mechanism. Free virus could enter the trophoblast cells by using alternative receptors, which included Fc receptors, galactosil ceramide and complement receptors. Indeed, some of these receptors have involved HIV-1 infection in neural cells and macrophages (Harouse et al., 1991; Lieberman et al., 1999; Robinson et al., 1988; Takeda et al., 1988).

Interestingly, we have found that HIV-1 infection of placental cells is much more efficiently produced by infected T-cell blasts than by free virus, in agreement with previous reports (Bourinbaiar and Nagorny, 1993; Douglas et al., 1991; Li and Burrell, 1992; Phillips and Tan, 1992; Sato et al., 1992). We further demonstrated that this effect was dependent on β2-integrin mediated leukocyte adhesion. Thus, antibodies to LFA-1 or to the common subunit of β2 integrins significantly prevented transfer of HIV-1 infection from T-cell blasts to placental cells. Besides, the inhibitory activity of these monoclonal antibodies correlated with their ability to block firm adhesion of T cells to placental monolayer. Our results are somewhat contradictory with a recent report that indicates that infected PBMC-placental cells contact favours transcytosis of HIV-1 but not infection of placental cells (Lagaye et al., 2001). The different cell lines used in both studies may explain the observed differences. However, we have seen HIV-1 infection in normal trophoblasts in addition to two transformed cell lines, thus supporting the ability of HIV-1 to infect cytotrophoblasts. Thus, HIV-1 entry into the placental trophoblast could be the result of either direct viral infection or the interaction between infected lymphocytes and trophoblast cells, although it is greatly enhanced in the latter case. It would be possible that adhesion between T-cell blast and trophoblast cells stimulates viral endocytosis or induces the expression of some surface molecules in placental cells, which increased virus entry.

In addition, infected placental cells can transfer infection to PBMC. This effect requires cell to cell contact and is also mediated by a LFA-1 dependent mechanism. Moreover, the rise in ICAM-1 in placental cells after HIV-1 infection may not only increase leukocyte adhesion and transfer of viral infection, but also allow the transmigration of PBMC through placenta to the fetus (Xiao et al., 1997). This fact may have some additional relevant consequences, since these maternal cells not only can transmit the virus but also they could trigger an immune response against the fetus. This hypothesis would be in agreement with the high percentage of miscarriage in HIV-1-infected women (Langston et al., 1995).

Previous studies have indicated the enhancing role of LFA-1 and ICAM-1, which are also expressed by virion themselves, on the initial processes of viral infection by favoring cell to cell contact (Hioe et al., 2001; Liao et al., 2000).

It was unclear whether placental cells contain virus in relative or absolute latency (Vicenzi and Poli, 1994). Latency is related with the absence of infectious virus but with the presence of proviral DNA integrated in the genomic cellular DNA (McCune, 1995). Besides, mature virus has been rescued from latent infection through coculture with susceptible cells or after cellular activation (David et al., 1992), also indicative of a latent state. Our results are in agreement with a relative latent state since proviral DNA could be detected and viral replication was more intense at early than at late times after infection and declining thereafter. The fact that HIV-1 replication could be induced upon cell to cell contact with PBMC also supports a latent state. In any case, the virus produced by placental infected cells was infectious, since placental infected cells can transfer the infection to T cells in cocultures.

In summary our results suggest that placental trophoblasts are actually infected by HIV-1 since this infection is mediated by a mechanism different from the use of the correceptors normally used by the virus to enter into T cells. More interestingly, infected T-cell blasts transfer the infection to placental cell much more efficiently than free virus. This effect depends on cell adhesion mediated by LFA-1 integrin. HIV-1 infection of placental cells enhances ICAM-1 expression and promotes leucocyte adherence to placental cells. Those placental cells may in turn pass the virus to T cell of the fetus again by a mechanism also involving LFA-1. Those results may help to explain vertical HIV-1 transmission in utero.

Materials and methods

Cells and virus stocks

Cytotrophoblasts were purified from normal term human placentas, after vaginal delivery, as described by Kliman et

al. (1986). Briefly, several cotyledons were minced and digested in a calcium- and magnesium-free Hanks' solution (CMF Hanks') containing 0.125% trypsin (Gibco, Paisley, Scotland, UK) and 0.2 μg/mL Dnase I (Sigma Chemical Co., St. Louis, MO). The supernatants were layered over fetal calf serum (FCS) (Biochrom KG, Seromed, Berlin, Germany) centrifuged at 2000 rpm for 10 min and the resultant pellets were resuspended in DMEM containing 25 mM HEPES and 25 mM glucose (DMEM-HG). This suspension was layered over a preformed discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) made up in Hanks' solution (from 70% to 5% Percoll in 5% steps). The gradient was centrifuged at 1.200 *g* at room temperature for 20 minutes. Cells sedimenting at densities between 1.051 and 1.063 specific gravity were collected. The density was determined by running parallel gradients loaded with density marker beads (Pharmacia).

Cytotrophoblasts were further purified by negative selection with anti-HLA antibodies. Immunomagnetic separation (Dynabeads, Dynal A.S, Oslo, Norway) was performed using monoclonal antibodies against human HLA-ABC (class I) and HLA-DP, DQ, DR (class II) to obtain purified cytotrophoblast (Lairmore et al., 1993). The purity of the isolated cells was confirmed by flow cytometry with anticytokeratin antibodies (Dako, AIS, Denmark). The purified cells were cultured in DMEM-HG 20% FCS, 2 mM L-glutamine (ICN Pharmacenticals, Costa Mesa, CA), 100 IU/ml penicillin and 100 μg/ml streptomicin (Gibco, Paisley, Scotland, UK) at a concentration of 10^6 cells/ml.

Transformed trophoblastic cell lines JAR (ATCC HTB 144) (Pattillo and Gey, 1968) and JEG-3 (ATCC HTB 36) (Kohler and Bridson, 1971) were routinely grown in RPMI (Biochrom KG seromed, Berlin, Germany) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine (ICN Pharmaceuticals), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Peripheral blood mononuclear cells (PBMC) were isolated from blood by Ficoll-Hipaque density gradient centrifugation (Pharmacia) as described (Munoz-Fernandez et al., 1996).

HIV-$1_{NL4.3}$ (X4), HIV-1_{BaL} (R5) established isolates or HIV-1_{2069I} (R5), and HIV-1_{1498I} (X4) primary clade B isolates from HIV-1 infected pregnant women were used. Production and titration of the viral stocks were as described (San Jose et al., 1998).

HIV-1 infection of placental cells

Trophoblasts, JAR, or JEG-3 cells were cultured in plates of different size depending of the experiments (Costar Corning, Cambridge MA). The various virus isolates obtained in our laboratory were added to individual cultures, at 0.1 m.o.i. and maintained for 4 h at 37°C. Then cultures were extensively washed with warm RPMI to remove all free virus (between 10 and 20 times). In those conditions we detected proviral DNA by nested PCR as previously described (Munoz-Fernandez et al., 1996). The placental cells were cultured in 10% FCS RPMI and virus titer and were quantified in the supernatant at various times after infection by p24 antigen production (INNOTEST HIV Antigen mAb, Innogenetics, Zwijndrecht, Belgium) or by the quantification of HIV-RNA (viral load) (Amplicor-HIV Monitor$_{TM}$ test, Roche, NJ). Mock infection was carried out with heat-inactivated (hi) viral preparations. Cell lines were trypsinized every three culture days, washed and plated at appropriate concentrations.

To block viral entry, JEG-3 cultures were preincubated 1 h at 37°C with the monoclonal antibodies anti-CD4, OKT4A and Leu3a (10 μg/ml) and with the chemokines MIP1-α, MIP-1β, RANTES, Eotaxin, SDF, and MCP-1 cytokines (1 μg/ml) (R&D Systems, Minneapolis, MN) or heparin sulfate (500 ng/mL) alone or in combination with some chemokines in 24 well plates before adding HIV-$1_{NL4.3}$ (0.1 m.o.i.) or HIV-1_{BaL} (0.1 m.o.i). Three days later viral load was quantified in culture supernatants.

Cocultures of PBMC and placental cells

To analyze the efficiency of HIV-transfer between T-cell blasts and trophoblast as well as adhesion, several different coculture assays were carried out.

For this PBMC from healthy HIV seronegative donors were isolated from whole blood by Ficoll Hypaque (Pharmacia) centrifugation basically as described (Munoz-Fernandez et al., 1996). T-cell blasts were obtaining by activation of PBMC (10^6/ml) in RPMI-1640 medium (BIOCHROM) supplemented with 10% fetal calf serum (FCS) with 2 μg/ml of PHA for 48 h at 37°C. Next they were incubated for 16 h with 40 U/ml of human IL-2. The cells were infected with 1 m.o.i HIV-1, for 2–4 h. Cells were washed with RPMI-1640 medium (BIOCHROM) supplemented with 10% FCS twice. Anti-human LFA-1 (CD11a), human LFA-2 (CD2), human β_2 integrins (CD18), human LFA-3 (CD58), all of them purchased from Endogen (USA) were added at a concentration of 50 μg/ml to the JAR cell line for 2 h where indicated. Then HIV-1 infected T-cell blasts (2×10^6) were added to JAR monolayers (10^6) and cocultured for 6 h at 37°C in presence of the corresponding antibodies. T-cell blasts were washed extensively (20 times) and with EDTA to remove all PBMC and cultures were incubated for 72 h at 37°C.

Transfer of HIV-1 from T cells to trophoblast cells

To study the adhesion of infected T cell to the trophoblast layer, T-cell blasts were labeled with [^{3}H]thymidine (10 μCi) for 16 h and washed prior to use in the assay. Thus, after culturing infected and labeled T-cell blasts as above, the cultures were washed 6 times to allow specifically interacting T cells to remain bound to the plates. Then the radioactivity in the remaining trophoblast cultures were evaluated and the number of T cells bound extrapolated by

determining in the control specific radioactivity per T-cell blast.

Transfer of HIV-1 from infected trophoblast cells to PBMC

Cocultures of infected placental cells (10^6) with T cell blast (2×10^6) were carried out as above with the cells in close contact for 6 h, or separated by 0.4 μm pore size transwells (Costar Corning, Cambridge MA) to avoid cell-cell interactions between both cellular types. Then the PBMC were collected by extensive washing and plating and at different days production of Ag p24 measured.

The adhesion of T-cell blast to infected JAR cells was evaluated by 3 independent methods. After 6 h of coculture in the presence of various antibodies where indicated, T-cell blast and JAR, the cultures were washed 6 times to remove nonadhered PBMC. Thus, the remaining cells bound to monolayers were evaluated by A) Normasky microscopy; B) fluorescence microscopy after staining with an anti-human CD45-FITC monoclonal antibody (JAR cells do not express CD45); or C) quantifying the amount of radioactivity bound to the plates. For this, T cells were labelled with [^{3}H]thymidine as mentioned above previous to addition to the plates. Radioactivity was measured in a scintillation counter, and the number of cells bound extrapolated.

Flow cytometry

Cell surface expression of CD4, CCR5 and CXCR4 in placental cells was evaluated by direct flow cytometry as previously described (Munoz-Fernandez et al., 1996). Briefly, placental cells were recovered by treatment of the monolayers with 0.02% EDTA-containing RPMI medium, and subsequently incubated with fluorescein isothiocyanate-labeled anti-CD4 Leu3a (Becton-Dickinson) and phycoeritrin anti-CXCR-4 (Pharmigen) monoclonal antibodies or with irrelevant fluorescein isothiocyanate or phycoerithin-labeled monoclonal antibodies as negative controls, for 30 min at 4°C. Then, the cells were washed in the above buffer and surface fluorescence was determined in a FACS-scan cytometer (Becton-Dickinson, Franklin Lakes, NJ). A minimum of 10,000 cells per point were analysed.

The purity of trophoblast suspension after magnetic negative selection was also evaluated by intracellular anti-cytokeratin staining using flow cytometry. For this, cells were washed with PBS twice and incubated 30 minutes at 4°C in PBS 2% paraformaldehide. After two washes in PBS they were incubated in 0.5% Twin PBS 15 minutes at 4°C in the dark. Cells were incubated with FITC-anti-cytokeratin antibody or with an irrelevant FITC bind antibody as negative control, and washed in 2% BSA PBS. Purity was greater than 97% in all experiments.

RT-PCR from placental cells

mRNA from 10^5 placental cells was isolated with oligo (dT) coated magnetic beads and subsequent reverse transcription (Series 9600, Promega, Corps., Madison, WI), according to the manufacturer's instructions. DNA amplification was performed with 5 or 10 μl of cDNA. GAPDH amplification was used as positive mRNA purification control. The following primers were used: CD4 sense (5′-GTGAACCTGGTGGTGATGAGAGC-3-′) and CD4 antisense (5′-GGGGCTACATGTCTTCTGAAACCGGTG-3′); CXCR4 sense (5′-GACCGCTACCTGGCCATT-3′) and CXCR4 antisense (5′-GTTGTAGGGCAGCCA-3′) (He, 1997); CCR3 sense (5′-TCCTTCTCTCTTCCTATCA-3′) and CCR3 antisense (5′-GGCAATTTTCTGCATCTG-3′) (He, 1997); and CCR5 sense (5′-AATCTTCTTCATCATCCTCC-3′) and CCR5 antisense (5′-TCTCTGTCACCTGCATAGC-3′) (He et al., 1997).

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